

AD _____

Award Number: DAMD17-99-1-9140

TITLE: Classical and Nonclassical Estrogen Receptor Action on
Chromatin Templates

PRINCIPAL INVESTIGATOR: Steven Nordeen, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health
Science Center
Denver, Colorado 80262

REPORT DATE: June 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

20001005 060

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 99 - 31 May 00)		
4. TITLE AND SUBTITLE Classical and Nonclassical Estrogen Receptor Action on Chromatin Templates		5. FUNDING NUMBERS DAMD17-99-1-9140		
6. AUTHOR(S) Steven Nordeen, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado Health Science Center Denver, Colorado 80262 E-MAIL: Steve.Nordeen@uchsc.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Improvement of hormone-based therapy in breast cancer and circumvention of its shortcomings is limited by the lack of detailed understanding of how steroids like estrogen work at a cellular and molecular level. The research supported by this award addressed the mechanism of action of estrogen action at its most fundamental level. Using newly-developed approaches, I investigated mechanisms of estrogen/estrogen receptor action on chromatin templates <i>in vitro</i> in order to better understand the role of chromatin in steroid-regulated gene expression. Specifically, experiments were proposed to assess the role of histone acetylation and high mobility group (HMG) chromatin proteins in estrogen receptor-directed transcription. Additionally, I sought to test whether <i>in vitro</i> transcription on chromatin templates could be used to address estrogen receptor action at nonclassical target genes. The chief accomplishment of these studies addressed the role of chromatin modification in activator-dependent transcription of chromatin templates. We found that the chromatin assembly extracts provided a critical factor and that acetyl CoA could restore activator-dependent transcription, implicating a critical role for chromatin modification in transcriptional regulation. However, other data suggested that acetyl CoA may account for only part of the stimulatory activity. Further experiments to address these findings are in progress.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 11	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A ___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A ___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

OK N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

OK N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature 6-19-88
Date

Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	
References.....	
Appendices.....	

INTRODUCTION: This award supported sabbatical studies directed to elucidating molecular mechanisms of estrogen receptor action. Newly developed technologies employing the assembly of chromatin to serve as template for estrogen receptor regulated transcription *in vitro* were used to address the aims outlined in the original application. These goals were to address the influence of high mobility group proteins on the regulation of transcription by estrogen receptor, the role of histone acetylation in estrogen receptor action, and the mechanisms of estrogen receptor action at promoters with nonclassical response elements. The purpose of these studies was to acquire sufficient expertise in the chromatin assembly/*in vitro* transcription technologies to be able to initiate studies of this sort in my own laboratory. Although the specific aims were quite ambitious, progress was made on all three aims. The summary that follows details this progress. Most importantly, insights were gained that has opened new research questions and sufficient expertise was acquired to permit the continuation of further studies in my own laboratory upon my return to my home institution.

ANNUAL SUMMARY: My sabbatical leave supported, in part, through this award was spent at the University of California, San Diego, Department of Biology in the laboratory of Jim Kadonaga. Dr. Kadonaga has a stellar reputation for his pioneering work on the role of chromatin in the regulation of gene expression. Recently his laboratory developed the first *in vitro* transcription system in which gene induction by a steroid receptor recapitulated *in vivo* biology, opening the door to mechanistic studies on steroid receptor action that are not possible in living cells. It was to become familiar with this system that I arranged to conduct studies in the Kadonaga laboratory.

Preparation of S190 chromatin assembly extracts. The chromatin assembly/*in vitro* transcription assay has many components, each of which must be carefully prepared and tested. One of the first endeavors I undertook was to prepare S190 chromatin assembly extracts. These extracts are at the heart of the transcription assay for it is the assembly of the DNA into chromatin template that permits the proper regulatory properties to be reconstructed *in vitro*. S190 extracts are prepared from 0-6 hour *Drosophila* (fruit fly) embryos beginning with about 120 grams of material. This is accomplished by harvesting embryos from a dozen or more population cages of flies every 6 hours for 48 hours. I prepared two batches of S190 extracts. To assemble a template for *in vitro* transcription the S190 extracts are first incubated with purified core histones for 30 minutes at room temperature. This is for the histone chaperone activity of the S190 extract. The actual assembly is accomplished during a subsequent 4-hour incubation at 27° C. The quality of the S190 extracts was assessed by analyzing the extent of the nucleosome array assembled on the plasmid template by micrococcal nuclease digests and plasmid supercoiling assays. Additional studies will be performed using these extracts in my laboratory at the University of Colorado Health Sciences Center.

Preparation of core histones. Assembly of plasmid templates into nucleosomal arrays requires the four core histones (H2A, H2B, H3, and H4). Core histones must be purified free from other nuclear proteins including linker histone H1. I purified core histones from *Drosophila* embryos using a procedure developed in the Kadonaga laboratory. This gave me my first experience using an FPLC for protein purification. The purity of the preparation was confirmed by SDS-polyacrylamide gel electrophoresis. As with the S190 chromatin assembly extracts, these

preparations of histones will be used in the additional studies that will be continued in my laboratory.

Preparation of HeLa cell transcription extracts. The transcription assay uses nuclear extracts from HeLa cells to provide many of the components of the transcriptional machinery. HeLa extracts were prepared beginning with a pellet of cells derived from 12 liters of HeLa cells. The procedure involves differential salt extraction and differential centrifugation. The preparation yielded about 5 milliliters of extract. The initial extract I made proved to be of poor quality and the preparation had to be repeated. The second preparation had good transcriptional activity. In addition to giving me experience with the preparation of HeLa extracts, these studies have provided the material to continue these studies upon returning to Colorado.

Preparation of tagged estrogen receptor and p300. Both of these proteins were overexpressed by infection of Sf9 insect cells with recombinant baculovirus vectors constructed in the Kadonaga laboratory. I purified estrogen receptor from extracts using FLAG affinity resins to selectively retain the FLAG tagged receptor. The coactivator p300 was expressed as a hexa-His tagged version and purified using commercial nickel resin. The addition of p300 to the transcription reaction greatly enhances the level of estrogen receptor-mediated induction of transcription. As above these preparations will continue to be of immediate use in continuing these studies.

Preparation of plasmids. Immediately before leaving Colorado I constructed two plasmids that I used in studies during the sabbatical leave. These two plasmids plus several others were purified from bacterial cell lysates by double banding over CsCl gradients in the laboratory at UCSD. These double-banded plasmids were used as templates for the studies that were performed and will continue to be of use in future studies.

In vitro transcription by estrogen receptor on classical and nonclassical templates. Once the necessary reagents were prepared I repeated previous work from the Kadonaga laboratory using the purified estrogen receptor to show ligand-dependence of estrogen receptor action on a classical, estrogen response element-containing promoter. Having done this, I tested whether estrogen receptor-mediated induction could be observed on a non-classical promoter where estrogen receptor interacts through a second protein instead of binding directly to sequence elements of the promoter. I chose the collagenase promoter for these studies. Estrogen receptor is thought to work by binding to the factor AP-1 which binds a site immediately upstream of the core collagenase promoter. Despite some hints that estrogen receptor-mediated induction could be seen, the effect was not large enough or consistent enough to believe. To see steroid receptor effects on the collagenase promoter probably is likely to require addition of purified AP-1 to the reaction as well. My laboratory will pursue this question further.

The effect of HMG proteins on transcriptional activation of chromatin templates by estrogen receptor. Additional studies addressed the role of high mobility group proteins 1 and 2 on estrogen receptor action. No effect of the addition of high mobility group proteins was observed on transcription. However, because these studies used crude S190 chromatin assembly extracts and HeLa extracts, it is likely that high mobility group proteins were already present. Additional efforts in this direction will require the use of purified chromatin assembly extracts and purified

transcription factors. It is now feasible to do such studies. Initial steps in this direction used purified chromatin assembly extracts and led to unexpected and interesting findings as detailed below.

In vitro transcription by progesterone receptor. Using purified progesterone receptor obtained from Dean Edwards at UCHSC and templates I made specifically for these studies, I demonstrated for the first time progesterone receptor-dependent transcription on a chromatin template. These studies also used for the first time with steroid receptors a natural promoter derived from the mouse mammary tumor virus.

Chromatin assembled by a recombinant assembly system fails to support activator dependent transcription in vitro: implications for a role for histone modification. Probably the chief accomplishment of my experimental endeavors was the unexpected demonstration that when a new, fully recombinant system was used to assemble the chromatin templates rather than an S190 extract neither estrogen receptor nor a synthetic transcription factor Gal4-VP16 could subsequently activate transcription. A series of studies showed that the effect of S190 was on a step subsequent to assembly. I speculate based on this and data discussed below that a remodeling of the chromatin template does not occur in the absence of S190. I showed further that much of the transcriptional activity could be restored by the addition of AcCoA. This is a particularly interesting finding since the role of acetylation of histones and other proteins is presently a topic of intense investigation. AcCoA is a substrate for the acetyltransferase enzymes that carry out the acetylation. However, close examination of several facets of my data suggest that S190 has an activity in addition to or instead of simply providing the AcCoA required for the activation of transcription. This subject will be pursued in my own laboratory. In addition, I plan to take advantage of my newly acquired expertise, reagents, and results to write grant applications to support pursuing these and other issues that can be addressed by exploiting the chromatin assembly/*in vitro* transcription system.

KEY RESEARCH ACCOMPLISHMENTS:

- Acquired the expertise to perform the techniques required for studies utilizing *in vitro* transcription by estrogen and other steroid receptors on chromatin templates.
- Prepared purified protein and functional extracts used for the described *in vitro* transcription studies as well as for use in future experiments that will build on the results obtained to this point.
- Evidence for *in vitro* transcription on natural promoter by the progesterone receptor.
- Evidence for a requisite role of Acetyl Coenzyme A in receptor-dependent and transcription factor-dependent transcription from chromatin templates.
- Additional data suggesting that factors in addition to Acetyl CoA may play a role in transcriptional regulation on chromatin templates.

REPORTABLE OUTCOMES:

1. Abstract poster presented at Keystone Symposium: Nuclear Receptors 2000 “Is chromatin modification or chromatin remodeling required for activator-dependent activation of transcription in vitro?” SK Nordeen, ME Levenstein, W Jiang, WL Kraus, JT Kadonaga
2. Abstract talk presented at Keystone Symposium: Nuclear Receptors 2000 “Chromatin structure and the Regulation of Transcription by RNA Polymerase II” X Huang, W Jiang, SK Nordeen, DV Fyodorov, ME Levenstein, V Alexiadis, JT Kadonaga
3. Presentation: The role of chromatin modification and chromatin remodeling in activator-dependent transcription. Talk given to Division of Medical Oncology, Univ. Colorado Health Sciences Center
4. Presentation: Chromatin remodeling and modification in the regulation of transcription by steroid receptors. Talk given to the Hormones and Cancer group of the University of Colorado Cancer Center
5. Presentation: The role of chromatin modification and chromatin remodeling in activator-dependent transcription. Annual Meeting of the Front Range Transcription Group.
6. Funding Applied for: NIH “Molecular Mechanisms of Steroid Hormone Action”

Chromatin Structure and the Regulation of Transcription by RNA Polymerase II

Xuejun Huang, Wen Jiang, Steven K. Nordeent†, Dmitry V. Fyodorov, Mark E. Levenstein, Vassilios Alexiadis, and James T. Kadonaga, Department of Biology, University of California, San Diego, La Jolla, CA 92093-0347, USA, and †Department of Pathology, University of Colorado Health Sciences Center, Denver, CO 80262, USA.

We have used a primarily biochemical approach for the analysis of the role of chromatin structure in the regulation of transcription by RNA polymerase II. In these experiments, we have found that the mechanism of transcriptional activation by factors such as the human estrogen receptor α can be recreated in vitro with chromatin templates but not with non-chromatin (naked DNA) templates. In previous work, however, we had used a crude extract from *Drosophila* embryos, termed the S-190, for the ATP-dependent assembly of chromatin (as periodic, extended nucleosome arrays) in vitro. Fortunately, in a separate project, we have been able to purify and to clone the factors in the S-190 extract that mediate the chromatin assembly reaction. Those studies have led to the development of a defined chromatin assembly system with purified recombinant ACF (ATP-utilizing chromatin assembly and remodeling factor) and purified recombinant dNAP-1 (*Drosophila* nucleosome assembly protein-1). It is relevant to note that ACF is a chromatin remodeling factor as well as a chromatin assembly factor. Thus, it has the capacity to mobilize nucleosomes, as is seen upon the binding of sequence-specific transcription factors in the regulation of gene activity. At the meeting, recent studies of transcription factor function with the newly-developed chromatin transcription system will be described.

361 Genetic Evidence for Relevance of TIF2 to Ligand-dependent Activation of Steroid Receptors
 Jun-ichi Nishikawa, Shigeki Arai, Shinichiro Yamachica, Kazuya Ogawa and Tsutomu Nishihara, Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka, Japan 565-0871

Members of the p160 coactivator family have been thought to play roles in mediating ligand-dependent transactivation of nuclear receptors. They have intrinsic HAT activity and may modify the chromatin organization of the target genes as well as act to link the receptors with the basal transcriptional apparatus. Although the p160 coactivators have been demonstrated to confer coactivation to nuclear receptors in the context of transient cotransfection experiments, their requirement in nuclear receptor transactivation function has not yet been definitively established. In order to assess the functional importance of TIF2, a member of the p160 coactivator family, we generated the TIF2-deficient cell by gene targeting using high homologous recombination- proficient chicken B lymphocyte line DT40. In the TIF2 null mutant, expression of SRC-1 mRNA was increased, although it is not expressed in wild-type DT40. While the disruption of TIF2 gene almost abolished the ligand-dependent activation of estrogen receptor, glucocorticoid receptor, it caused only partial effect on vitamin A receptor, vitamin D receptor and thyroid hormone receptor. The steroid receptor mediated transcriptional activation was recovered by ectopic expression of SRC-1 as well as TIF2, suggesting possible physiological redundancy of coactivators.

363 Is chromatin modification or chromatin remodeling required for activator-dependent activation of transcription in vitro?

Steven K. Nordeen*, Mark E. Levenstein, Wen Jiang, W. Lee Kraus, James T. Kadonaga. *Dept. of Pathology, Univ. Colorado Health Sciences Center, Denver, CO 80262 and Dept. of Biology, Univ. California San Diego, La Jolla, CA 92093

In vitro transcription of templates that have been assembled into nucleosomal templates by the addition of core histones and an ATP-dependent assembly extract from *Drosophila* embryos (S190) is dependent on transcriptional activators. The histone chaperone and chromatin assembly activities of the S190 extracts have been isolated, cloned, and expressed as purified proteins. Estrogen receptor or Gal4-VP16 fail to activate transcription on templates assembled into chromatin by the recombinant system. The addition of Acetyl CoA before or after chromatin assembly restores activator-dependent transcription on templates assembled by the recombinant system suggesting that chromatin modification or remodeling is required for transcriptional activation. A wide range of Acetyl CoA concentrations is effective at restoring transcription but this effect can be largely abolished by the addition of an Acetyl CoA depletion system.

362 Robert M. Nissen & Keith R. Yamamoto
 Department of Cellular and Molecular Pharmacology, PIBS Biochemistry and Molecular Biology Program, University of California at San Francisco, San Francisco, CA 94143-0502

Glucocorticoids are a class of steroid hormone that have been used for the treatment of inflammatory diseases for decades, and glucocorticoids are potent inhibitors of many pro-inflammatory genes, including the IL8 gene. Research on the mechanism by which glucocorticoids act has yielded several critical observations. In the absence of hormone, the glucocorticoid receptor (GR) is localized to the cytoplasm. Upon hormone binding, GR translocates to the nucleus where it can then regulate transcription, and it has long been known that GR regulates transcription in a context dependent manner. The context dependence of GR gene regulation has led to the classification of glucocorticoid response elements (GREs): simple GREs are DNA elements that GR binds directly without auxiliary factors; composite GREs also involve direct DNA binding, but the regulatory outcome depends on the composition of a neighboring transcription factor; and tethering GREs are DNA elements that GR does not bind to at all, but instead code the DNA binding sites of other known transcription factors, namely, AP-1 and NF-kappaB. The GR inhibits the pro-inflammatory transcription factor NF-kappaB by specifically targeting the RelA/p65 subunit. Binding of GR to DNA is not involved in RelA inhibition, and Ikb-alpha induction is absent from numerous cell types capable of supporting the inhibitory effects of glucocorticoids. Consistent with this, inhibition is independent of new protein synthesis. Interestingly, several groups have demonstrated a physical association between GR and the RelA subunit, and this has led to speculation that a ternary complex between GR and RelA might exist on NF-kappaB response elements in vivo. Using a combination of techniques including the chromatin immunoprecipitation assay, the aim of this work is to determine the mechanism of GR regulation of NF-kappaB at the IL-8 gene in the human lung cell line A549 and to define the role of physical association, in vitro and in vivo. Steps taken toward this goal include mapping the physically and functionally interacting regions between GR and RelA, and demonstrating that the two proteins can interact directly in vitro via their DNA binding domains. Inhibition of RelA requires a GR domain outside its interacting region, but the interacting region is sufficient to target RelA in vivo. Using the chromatin immunoprecipitation assay at the IL-8 promoter, we have shown that RelA remains chromatin associated during glucocorticoid inhibition and we have examined the fate of RNA polymerase II during inhibition.

364 Inhibition of tamoxifen activated estrogen receptor transcriptional activity by a putative RNA binding protein.

John D. Norris, Daju Fan and Donald P. McDonnell, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710

The biological actions of estrogens and antiestrogens are manifest through two distinct high affinity receptors, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). ER α contains two dominant transcriptional activation domains, activation function 1 (AF-1) which resides in the receptor N-terminus and activation function 2 (AF-2) which is contained within the receptor C-terminus. It has now become clear that the ability of ER α to interact differentially with co-activators and co-repressors is a primary determinant of estrogen and antiestrogen pharmacology. Recently, several proteins have been identified that interact with the AF-2 domain in a ligand-dependent manner and have been shown to co-activate ER α transcriptional activity. Presently, the role of the N-terminal domain in ER α signaling is not well defined. We report here the isolation of a protein that interacts with the N-terminus of ER α in a ligand-independent manner. This protein contains a consensus RNA binding domain and shares homology with several known RNA binding proteins. Subsequent studies revealed that this protein interacts with ER β but not progesterone receptor B (PR-B) or thyroid receptor beta (TR β). This protein appears to be localized to the nucleus and northern analysis revealed that it is highly expressed in the heart and skeletal muscle and poorly expressed in the liver. Surprisingly, expression of this protein leads to repression of tamoxifen-mediated transcription while minimally affecting estradiol mediated transcription suggesting this protein may be a novel co-repressor of ER transcriptional activity. [Supported by DK48807]